lysozyme in this combined therapy is unclear. It may only potentiate the activity of the antibiotics and have no direct effect on the pneumonia iteself. One skilled in the art cannot conclude that lysozyme per se may be used to treat pneumonia.

Please note the following referenced remarks in support of the applicants' arguments:

A reference may be said to teach away when a person of ordinary skill, upon reading the reference, would be led in a direction divergent from the path that was taken by the applicant [In re Vaeck, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991)].

A prior art reference must be considered in its entirety, i.e., as a whole, including portions that would lead away from the claimed invention. W.L. Gore & Associates, Inc. v. Garlock, Inc., 721 F.2d 1540, 220 USPQ 303 (Fed. Cir. 1983), cert. denied, 469 U.S. 851 (1984).

The rationale for not using lysozyme as an agent for treating pneumonia is based on a number of earlier studies demonstrating that this protein has only limited antibacterial activity in vitro (e.g. Neu et al. Effect of human lysozyme on gram-positive and gram-negative bacteria. Antimicrob. Agents Chemother. 8:442, 1968). Various strategies have been developed to circumvent this limitation. In particular lysozyme has been combined with various antibiotics to treat pneumonia (e.g. Luniakin et al). Only recently has it been shown that lysozyme may interact in vivo with other endogenous antimicrobial peptides, resulting in a much more potent bacteriocidal activity than previously suspected (see enclosed reference by Akinbi et al which postdates the filing of the provisional application

Please note the following referenced remark:

Evidence of unexpected results must be weighed against evidence supporting prima facie obviousness in making a final determination of the obviousness of the claimed invention. In re May, 574 F.2d 1082, 197 USPQ 601 (CCPA 1978)

As additional evidence of the nonobviousness of the applicants' claims, we again refer to the enclosed reference by Akinbi et al. In para 1, column 1, p 5760, the authors state:

"Despite numerous studies confirming the antibacterial properties of lysozyme in vitro, ***there are no studies that have directly assessed the role of lysozyme in the killing of lung pathogens in vivo****."*

Furthermore (p 5764, column 1, para 3):

"The spectrum of lysozyme antimicrobial activity in vitro appears to be relatively narrow, ***leading some investigators to conclude that exogenous lysozyme would be of little benefit in controlling bacterial infection****."*

Please note the following referenced remark:

The totality of the prior art must be considered, and proceeding contrary to accepted wisdom in the art is evidence of nonobviousness [In re Hedges, 783 F.2d 1038, 228 USPQ 685 (Fed. Cir. 1986)].

2) The Examiner rejects claims 28, 29, 32-34 under USC 102(b) as being anticipated by Weaver et al, stating that "Weaver teaches administering

one will also treat pneumonia." For the reasons presented below, reconsideration of the rejection is respectfully requested.

Weaver does not teach administering lysozyme to treat cystic fibrosis or pneumonia associated with cystic fibrosis. Weaver teaches the use of a lysozyme fusion protein **as a substitute for lysozyme**, because of the possible toxicity associated with in vivo use of native lysozyme. This clearly differentiates his art from the applicants' claims. One skilled in the art cannot conclude from Weaver et al that lysozyme per se may be used to treat pneumonia.

The nonobviousness of the applicants' claims is also supported in Weaver et al (column 2, lines 18-22), which states:

"While the antibacterial effects of lysozyme in vitro have been well documented, there has heretofore been no way to exploit these effects of lysozyme ***for in vivo use.*** *Previous reports furthermore implied that* ***sustained lysozyme administration would be harmful."***

Claim Rejections Under 35USC § 103

1) The Examiner rejects claims 28-35 under 35 USC 103(a) as being unpatentable over Weaver et al and Gavrilenko et et al, taken with with Kats et al. The rejection is a matter of record. Reconsideration of the rejection is respectfully requested, based on the applicants' arguments of record, which are reprised below.

use of lysozyme to treat pneumonia" it is stated in column 2 lines 19-22 that

"While the antibacterial effects of lysozyme in vitro have been well documented, there has heretofore been no way to exploit these effects of lysozyme for in vivo use. Previous reports furthermore implied that sustained lysozyme administration would be harmful."

Clearly, Weaver et al are teaching away from the use of lysozyme alone as an agent to treat pneumonia. Indeed, the whole point of the invention described in Weaver et al is to **circumvent any potentially harmful effects of administering native lysozyme** by fusing it with surfactant protein-B. Therefore, the Examiner cannot state that the use of lysozyme to treat pneumonia is made obvious by this reference.

With regard to Gavrilenko et al, the Examiner states that:

"Further, as noted by Gavrilenko, lysozyme is used to treat chronic bronchitis which also leads to pneumonia."

There is no support in the medical literature for the notion that chronic bronchitis leads to pneumonia. Bronchitis (acute or chronic) and pneumonia are two different diseases with different etiologies. Chronic bronchitis is a component of Chronic Obstructive Lung Disease, which encompasses bronchitis, asthma, and emphysema. It involves the upper airways of the respiratory tract and is commonly due to cigarette smoking or exposure to air pollutants, which induce an inflammatory reaction. The Examiner's argument that "...lysozyme is used to treat chronic bronchitis which also leads to pneumonia" is therefore

Furthermore, with regard to the Examiner's continued use of the previously recorded prior art (Weaver et al., Gavrilenko et al., Kats et al, he has not considered the applicants' previous arguments (listed above) against the use of these references as prior art. The Examiner is required to provide a rationale for his continued use of these references over the stated objections of the applicants.

When an applicant submits evidence, whether in the specification as originally filed or in reply to a rejection, the examiner must reconsider the patentability of the claimed invention. The decision on patentability must be made based upon consideration of all the evidence, including the evidence submitted by the examiner and the evidence submitted by the applicant. A decision to make or maintain a rejection in the face of all the evidence must show that it was based on the totality of the evidence [In re Eli Lilly & Co., 902 F.2d 943, 14 USPQ2d 1741 (Fed. Cir. 1990)].

Furthermore,

Evidence traversing rejections must be considered by the examiner whenever present. All entered affidavits, declarations, and other evidence traversing rejections are acknowledged and commented upon by the examiner in the next succeeding action. The extent of the commentary depends on the action taken by the examiner. Where an examiner holds that the evidence is sufficient to overcome the prima facie case, the comments should be consistent with the guidelines for statements of reasons for allowance. Where the evidence is insufficient to overcome the rejection, the examiner must specifically explain why the evidence is insufficient. General statements such as "the declaration lacks technical validity" or "the evidence is not commensurate with the scope of the claims" without an explanation supporting such findings are insufficient (MPEP

2) The Examiner states that "the language 'consisting essentially of' is interpreted to mean 'comprising' unless applicant can show that the other ingedients in the references would materially change the fundamental characteristics of applicant's invention"

In the face of growing human resistance to antibiotics, there is an unmet need for the use of alternative compounds to treat pneumonia (see enclosed references by Hancock et al and Yan et al). The novel aspect of the applicants' claims is the use of lysozyme per se as an **alternative** (not an adjunct) to antibiotics for the treatment of pneumonia. Inclusion of additional steps materially changes the characteristics of the applicant's invention. In particular, the use of antibiotics in conjunction with lysozyme as indicated by Luniakin is contrary to the thrust of the applicants' claims. The substitution of a lysozyme fusion protein for native lysozyme as taught by Weaver et al also runs counter to the applicants' teaching.

3) The Examiner rejects claims 28-35 under 35 USC 103(a) as being unpatentable over Weaver et al or Luniakin et al, stating that "Both references each teach that lysozyme is administered to a patient having pneumonia or inherently has pneumonia as taught above in Weaver. To use lysozyme from chicken eggs or produced by fermentation, etc. is simply the choice of the artisan."

The applicants' claims are not rendered obvious by either Weaver et al or Luniakin et al for the reasons presented above. Reconsideration of the rejection is therefore respectfully requested.

In summary, it is respectfully requested that these claims now be placed in condition for allowance. Where appropriate, the applicants request constructive assistance with regard to the wording of the claims in order to place them in such condition.

Sincerely yours,

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Bacterial Killing Is Enhanced by Expression of Lysozyme in the Lungs of Transgenic Mice[1]

Henry T. Akinbi,[2] Ralph Epaud, Hetal Bhatt, and Timothy E. Weaver

To assess the role of lysozyme in pulmonary host defense in vivo, transgenic mice expressing rat lysozyme cDNA in distal respiratory epithelial cells were generated. Two transgenic mouse lines were established in which the level of lysozyme protein in bronchoalveolar (BAL) lavage fluid was increased 2- or 4-fold relative to that in WT mice. Lung structure and cellular composition of BAL were not altered by the expression of lysozyme. Lysozyme activity in BAL was significantly increased (6.6- and 17-fold) in 5-wk-old animals from each transgenic line. To determine whether killing of bacteria was enhanced by expression of rat lysozyme, 5-wk-old transgenic mice and WT littermates were infected with 10^6 CFU of group B streptococci or 10^7 CFU of a mucoid strain of *Pseudomonas aeruginosa* by intratracheal injection. Killing of group B streptococci was significantly enhanced (2- and 3-fold) in the mouse transgenic lines at 6 h postinfection and was accompanied by a decrease in systemic dissemination of pathogen. Killing of *Pseudomonas aeruginosa* was also enhanced in the transgenic lines (5- and 30-fold). Twenty-four hours after administration of *Pseudomonas aeruginosa*, all transgenic mice survived, whereas 20% of the WT mice died. Increased production of lysozyme in respiratory epithelial cells of transgenic mice enhanced bacterial killing in the lung in vivo, and was associated with decreased systemic dissemination of pathogen and increased survival following infection. *The Journal of Immunology*, 2000, 165: 5760–5766.

Clearance of pathogens in the respiratory tract is mediated at least in part by the synthesis and secretion of host defense molecules into the airway lumen. One of the most abundant antimicrobial proteins in the lung, lysozyme, is synthesized and secreted by glandular serous cells, surface epithelial cells, and macrophages in the human airway (1). Human lysozyme (muramidase, *N*-acetyl muramide glycanohydrolase, EC 3.2.1.17) is a cationic protein of 148 aa that cleaves glycosidic bonds of *N*-acetyl-muramic acid, damaging the bacterial cell wall and ultimately killing the organism by lysis (2). Bacteriolytic assays indicate that purified lysozyme is active against some Gram-positive bacteria, but has relatively little activity against Gram-negative bacteria, largely because the outer membrane of these organisms limits access of the enzyme to its substrate (3). The concentration of lysozyme in human airway surface liquid ranges from 20–100 μg ml, a concentration sufficient to kill two important airway pathogens, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, in vitro (4). Despite numerous studies confirming the antibacterial properties of lysozyme in vitro, there are no studies that have directly assessed the role of lysozyme in the killing of lung pathogens in vivo. The current study was therefore undertaken to evaluate the impact of constitutively elevated levels of lysozyme on lung structure and pathogen clearance in transgenic mice.

The spatial expression of pulmonary lysozyme in rodents differs from that in humans in that the enzyme is principally synthesized and secreted by type II alveolar epithelial cells (5, 6). Lysozyme is one of the most abundant proteins in rat bronchoalveolar lavage (BAL)[3] fluid, accounting for as much as 6–7% of the total soluble protein (5). Although two lysozyme genes have been identified in the rat, only the type I gene is expressed at detectable levels (7); in contrast, two lysozyme genes are expressed in the mouse, lysozyme M and lysozyme P (8, 9). Lysozyme M is the major form expressed in the lung, and only very low levels of lysozyme P mRNA are detected in mouse lung (8). In the current study rat lysozyme type I, the homologue of mouse lysozyme M, was cloned under control of the human surfactant protein C (SP-C) promoter to direct expression of the transgene to the distal respiratory epithelium (10–12). Transgenic mice expressing rat lysozyme exhibited significantly enhanced antimicrobial activity, including enhanced bacterial killing, decreased systemic dissemination of pathogen, and increased survival following infection.

Materials and Methods

Generation of transgenic mice expressing rat lysozyme

The rat lysozyme cDNA was generated from rat type II cell RNA by RT-PCR using upstream primer 5′-GAA TTC ATG AAG GCT CTC CTA GTT CTG and downstream primer 5′-GAA TTC TCA GAC TCC GCA GTT CCG CAA TAT. The amplified 400 bp fragment was confirmed to be rat lysozyme by sequence analysis and was cloned into the *Eco*RI site of BGI-hSPC vector, which contained the 3.7 kb human SP-C promoter, rabbit β globin intron 2, and bovine growth hormone polyadenylation signal (13, 14). The transgene DNA construct was excised from the PUC18 vector backbone by *Not*I-*Xho*I digestion, gel purified using Qiaex resin (Qiagen, Germany), dialyzed for 48 h against 5 mM Tris (pH 7.4)-1 mM EDTA, and microinjected into fertilized FVB/N oocytes by the Children's Hospital Transgenic Core facility. Founder mice were identified by a transgene-specific PCR with primers that amplified a 310-bp fragment spanning the junction of the rabbit β-globin intron and the rat lysozyme cDNA: upstream primer, 5′-AAT TCT GGC TGG CGT GGA AA; downstream primer, 5′-TTG GTA GGG ATC CCC AAG GCA TT. PCR conditions were 28 cycles at 55°C (myeloperoxidase-negative cells) [illegible] μM [illegible]

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Cetus, Foster City, CA), and 1× AmpliTaq reaction buffer. PCR results were verified by Southern analyses using a ^{32}P-labeled probe that hybridized to the rabbit β-globin intron. For all studies, transgenic mice were compared with wild-type (WT) littermate controls. All mice used in this study were housed in pathogen-free rooms in the animal facility of Children's Hospital Medical Center (Cincinnati, OH).

Characterization of transgenic mice

RNA (RT-PCR) analysis. To identify lines that expressed rat lysozyme mRNA, lung tissues were collected from 5- to 6-wk-old transgenic mice and control WT littermates. Total cellular RNA was isolated using TRIzol reagent (Life Technologies, Grand Island, NY). One microgram of DNase I-treated RNA was reverse transcribed using the Superscript kit (Life Technologies) with oligo(dT) as primer. For RT-PCR analyses, rat lysozyme cDNA-specific primers (upstream, 5′-ACA CAA GCC AGA AAC TAC AACC; downstream, 5′-GAA TTC TCA GCA TCC GCA GTT CCG AAT) were used with the PCR conditions described above. β-Actin was coamplified in the PCR reaction as an internal control.

Northern analysis. Ten micrograms of total RNA isolated from lung tissues of 5-wk-old transgenic mice and control WT littermates was fractionated by gel electrophoresis, blotted onto a nylon membrane, probed with biotin-labeled rat or mouse lysozyme cDNA (provided by Dr. Rainer Renkawitz, Max Planck Institut für Biochemie, Martinsried, Germany), and detected by chemiluminescence (Pierce, Rockford, IL).

Analysis of protein expression. To identify transgenic mouse lines that expressed rat lysozyme protein, Western blotting was performed using a rabbit anti-human lysozyme Ab (Accurate Chemicals and Scientific Corp, Westbury, NY) that cross-reacts with both mouse and rat lysozyme (M_r ~15K). Lungs were isolated from 5-wk-old offspring of transgenic founders and homogenized in PBS with 1% (v/v) protease inhibitor mixture (Sigma, St. Louis, MO). Protein concentration was determined by bicinchoninic acid protein assay (15). One-half microgram of total lung protein was analyzed by SDS-PAGE and Western blotting, as previously described (16). To assess the level of secreted lysozyme, BAL fluid was obtained from six 5-wk-old transgenic mice and five control WT littermates as described below. One-half microgram of total protein of BAL fluid was resolved by SDS-PAGE and Western blotted with rabbit anti-human lysozyme Ab. Levels of lysozyme proteins in lung homogenates and BAL fluids were quantitated by scanning densitometry using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

BAL fluid cell count. Five 5-wk-old transgenic mice and five control WT littermates were exsanguinated by transecting the abdominal aorta and the inferior vena cava after a lethal i.p. injection of sodium pentobarbital. The lungs were lavaged three times with 1-ml aliquots of PBS. BAL fluid was centrifuged at 2000 × *g* for 10 min, and the pellet was resuspended in 0.5 ml of PBS. A 50-μl aliquot was stained with an equal volume of 0.4% trypan blue (Life Technologies) for total cell count on a hemocytometer. Differential cell counts were made on cytospin preparations stained with Diff-Quik (Scientific Products, McGraw Park, IL).

Lysozyme enzyme activity assay. To assess lysozyme enzyme activity, BAL fluid was obtained from 5-wk-old transgenic mice and control WT littermates. BAL fluid containing 1 μg of total protein was incubated with killed *Micrococcus lysodeikticus* suspended in 0.4 M phosphate buffer, pH 6.7, at an OD (450 nM) of 1 at 37° C. The change in OD was assessed after 1 h of incubation (37° C). Purified chicken egg lysozyme was used to generate a standard curve (1 U of enzyme activity equals a 0.001 change in OD).

Lung morphology and immunohistochemistry. To assess lung morphology, lungs from four 5-wk-old transgenic mice and four control WT littermates from transgenic line 3.5 were inflation-fixed for immunostaining and light microscopy as previously described (15). Immunostaining for lysozyme was performed using antiserum directed against human lysozyme (Accurate Chemicals and Scientific Corp.). Parallel lung sections were incubated with normal rabbit serum to verify the specificity of immunostaining.

Bacteria killing assays

Group B streptococci (GBS). Stock cultures of GBS used in this study were clinical isolates provided by Dr. J. D. Wessels (D.J. [illegible]

CFU/100 μl. The concentration of the inoculum was verified by quantitative culture on sheep blood agar plates.

Pseudomonas aeruginosa. The strain of *P. aeruginosa* was a mucoid segregant isolated from the sputum of a cystic fibrosis patient (provided by Dr. D. J. Hassett, University of Cincinnati College of Medicine, Cincinnati, OH). *P. aeruginosa* obtained from a single passage was stored in aliquots at −70° C in 20% glycerol Luria-Bertoni (LB) broth. For each experiment, an aliquot of bacteria was plated on LB agar followed by inoculation into 4 ml of LB broth. Preparation of the inoculum was conducted as described for GBS; the concentration (CFU) of the inoculum was verified by quantitative culture on LB plates.

Bacterial infection. The dose of bacteria selected for these studies was based on the results of previous studies in the FVB/N mouse strain (19). Mice were anesthetized with isofluorane, the trachea was exposed through an anterior midline incision, and 10^6 CFU (GBS) or 10^7 CFU (*P. aeruginosa*), suspended in 100 μl of sterile PBS, was delivered just beneath the cricoid cartilage. The incision was sealed by applying one drop of surgical glue (Nexabrand, Veterinary Products Laboratories, Phoenix, AZ). As a control, 100 μl of nonpyrogenic PBS was similarly instilled into transgenic and WT mice. To assess bacterial load at 6 h (GBS) or 24 h (*P. aeruginosa*) postinfection, mice were anesthetized with i.p. pentobarbital and exsanguinated by transecting the abdominal aorta, and lung and splenic tissues were harvested, weighed, and subsequently homogenized in sterile PBS. Serial dilutions of homogenates were plated on blood agar (GBS) or LB (*Pseudomonas*) plates and incubated at 37° C overnight. Viable pathogen counts in the lung and spleen were estimated from the number of colonies after 24 h of quantitative culture and expressed as CFU per gram of tissue.

To determine survival following infection, 20 5- to 6-wk-old transgenic mice from the 3.5 line and 20 WT littermates were infected with 2×10^7 CFU *P. aeruginosa* intratracheally, as described above. Water and food were provided ad libitum during the period of observation. The number of surviving mice was counted every 12 h for up to 60 h, at which time all surviving mice were sacrificed.

Cytokine levels following infection with P. aeruginosa

Eight 5- to 6-wk-old transgenic mice and eight age-matched WT littermates were infected intratracheally with 10^7 CFU of *P. aeruginosa* suspended in 100 μl of PBS. Mice were sacrificed at either 6 or 24 h, lungs were lavaged as described above, and lung tissues were homogenized in 1 ml of PBS with 1% (v/v) protease inhibitor mixture; the total volume after homogenization was adjusted to 2 ml with PBS. Mouse TNF-α, IL-6, macrophage inflammatory protein-2 (MIP-2), and RANTES were measured by sandwich enzyme immunoassay (R&D Systems, Minneapolis, MN). The limits of sensitivity for each cytokine are >5.1, <3.1, 1.5, and >2 pg/ml, respectively. All samples were assayed in duplicate, and values were normalized to total protein in the samples.

Statistical analyses

Differences between groups were assessed by one-way ANOVA, and differences between means were assessed by contrast comparisons and the Student-Newman-Keuls test (StatView, Abacus Concepts, Berkeley, CA). Data are expressed as the mean ± SD. Nonparametric survival distributions were estimated to examine differences in survival of transgenic mice and WT littermates. The differences between the two groups were analyzed using Kaplan Meier curve statistics.

Results

Generation of transgenic mice

To assess the role of lysozyme in pulmonary host defense, transgenic mouse lines were generated in which rat lysozyme was targeted to the distal respiratory epithelium under the direction of the 3.7-kb human SP-C promoter. Seven of 21 offspring from fertilized oocyte injections were positive for the transgene, as assessed by PCR and confirmed by Southern blot analyses of tail DNA (not shown). Transgenic offspring were indistinct from WT littermates with respect to body weight, lung weight, longevity, and repro-

[illegible] Northern blot analysis of total RNA isolated from the lungs of 5-wk-old

mice. A cDNA probe specific for the rat lysozyme transgene detected an approximately 1-kb transcript (Fig. 1*A*). When the same blot was probed with a mouse lysozyme cDNA, both the larger endogenous mouse lysozyme mRNA and rat lysozyme transgene mRNA were detected.

Because rat and mouse lysozyme have very similar m.w., it was not possible to distinguish the two proteins by SDS-PAGE. Total levels of lysozyme (rat and mouse) were estimated by Western blotting of equal amounts of protein from lung homogenates or BAL fluid from 5-wk-old transgenic mice and WT littermates. Total lysozyme protein in mice from transgenic line 2.6 was increased 2-fold in both lung homogenate (Fig. 1*B*) and BAL fluid (not shown); total lysozyme protein in mice from transgenic line 3.5 was increased 4-fold compared with that in control WT littermates (Fig. 1*B*). Lysozyme (M_r 142) was not detected when lung homogenates and BAL were blotted with nonimmune rabbit serum.

To determine whether increased lysozyme protein levels were associated with increased enzyme activity, lysozyme enzyme activity in BAL fluid from 5-wk-old transgenic mice and wild-type littermate controls was assessed by a turbidimetric assay using purified chicken egg lysozyme as a standard. Lysozyme enzyme activity in mice from transgenic line 3.5 was increased 17.7-fold compared with that in wild-type mice (550 U ng BAL protein vs 31 U ng BAL protein in WT mice; $p = 0.0001$; Fig. 2). As predicted from Western blot analyses, lysozyme enzyme activity in BAL from line 2.6 was increased relative to that in WT controls ($p = 0.02$), but was less than that in BAL from transgenic line 3.5.

The pattern of lysozyme expression was characterized by immunostaining of paraffin-embedded lung sections from 5-wk-old transgenic mice and control WT littermates. In WT mice, immunostaining of endogenous lysozyme was restricted to alveolar epithelial cells, whereas in transgenic mice, lysozyme staining was equally prominent in nonciliated bronchiolar cells and alveolar epithelial cells (Fig. 3). No staining was detected in the submucosa or in nonepithelial cells in either WT or transgenic mice or when parallel lung sections from transgenic mice were incubated with nonimmune rabbit serum. Lysozyme expression in transgenic mice therefore conforms to the previously reported expression pattern for the 3.7-kb human SP-C promoter (10–12).

The histologic features of paraffin-embedded, hematoxylin eosin-stained sections of lungs from uninfected 5-wk-old transgenic mice and control WT littermates were also compared. The architecture of lungs from transgenic mice were indistinct from that of lungs from littermates with respect to septal thickness, type II cell hypertrophy, and hyperplasia (not shown). There was no evidence of pulmonary edema or vascular congestion, and inflammatory cells were not detected in lung sections from uninfected transgenic mice. In addition, there were no differences in the total cell count or the distribution of cell types recovered from BAL fluid.

Effect of transgene expression on killing of bacteria in infected mice

Killing of GBS. To determine whether increased lysozyme levels in the airway enhanced killing of bacteria in the lungs, quantitative cultures of lung homogenates from transgenic mice and WT littermate controls were compared following intratracheal injection

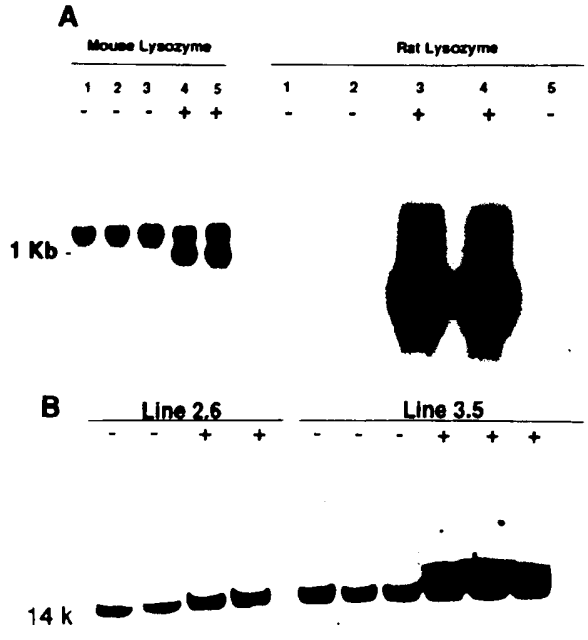

FIGURE 1. Expression of rat lysozyme in transgenic mice. *A*, Representative Northern blot analysis of 10 μg of total cellular RNA from lung tissue isolated from 5-wk-old transgenic mice from line 3.5 (*lanes 4* and *5*, *left panel*; *lanes 3* and *4*, *right panel*) and WT littermates (*lanes 1–3*, *left panel*; *lanes 1*, *2*, and *5*, *right panel*). Samples in the *left panel* were probed with the labeled mouse lysozyme cDNA, which detected both mouse lysozyme and the rat lysozyme transgene (1 kb, indicated by the arrow). Positions of 28S and 18S ribosomal RNA are indicated. *B*, Western blot analysis of lysozyme (M_r 14 kDa, indicated by the arrow) in lung homogenates from transgenic mice from lines 2.6 and 3.5 and WT littermates. Positions of molecular weight markers are indicated.

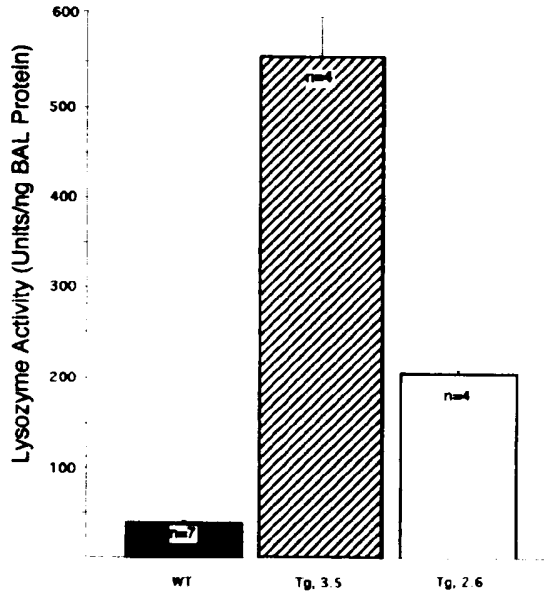

FIGURE 2. Lysozyme activity in BAL fluid from WT and transgenic mice. Purified chicken egg white lysozyme was used to generate a standard curve. Data are the mean ± SEM. *n*, number of animals per experiment.

FIGURE 3. Lysozyme protein is expressed in alveolar cells and bronchiolar epithelial cells in transgenic mice. Cellular localization of lysozyme protein in transgenic mice was assessed by immunohistochemistry on lung sections from 5-wk-old transgenic mice from line 3.5 (*C*) and WT littermates (*B*) using anti-human lysozyme Ab. No staining was detected when parallel sections from transgenic mice were incubated with nonimmune rabbit serum (*A*). Lysozyme was detected in alveolar epithelial cells (arrow) in WT and transgenic mice. Lysozyme expression was also detected in bronchiolar epithelial cells of transgenic mice, but not WT mice (arrowheads). Magnification, ×240.

of 10^6 CFU of GBS. All mice survived until sacrifice at 6 h postinfection. Killing of GBS was enhanced 3.2-fold in mice from transgenic line 3.5 ($2.1 \pm 0.1 \times 10^6$ CFU g protein vs $6.8 \pm 0.5 \times 10^6$ in WT mice; $p = 0.01$; Fig. 4). The incidence of systemic dissemination of infection, as assessed by growth of GBS on plates inoculated with splenic homogenates, was less in transgenic mice (27 vs 60%; $p = 0.04$). Killing of GBS was enhanced 1.7-fold in mice from transgenic line 2.6 ($4.2 \pm 0.8 \times 10^6$ CFU g protein vs $7.1 \pm 0.6 \times 10^6$ in WT mice; $p = 0.04$).

***Killing of* P. aeruginosa.** All transgenic mice survived until sacrifice at 24 h postinfection following intratracheal infection with 10^7 CFU of *P. aeruginosa*; in contrast, 20% of infected WT mice died. Bacterial killing was enhanced 30.6-fold in mice from transgenic line 3.5 ($1.06 \pm 0.05 \times 10^6$ CFU g protein compared with $32.4 \pm 0.41 \times 10^6$ in WT littermate controls; $p = 0.03$; Fig. 5). Bacterial killing was enhanced approximately 6.2-fold in mice from transgenic line 2.6 ($6.52 \pm 0.71 \times 10^6$ CFU g protein; $p = 0.05$). Systemic dissemination was not detected in surviving mice at 24 h postinfection. Following intratracheal infection with 2×10^7 CFU of *P. aeruginosa*, the mean survival time for transgenic mice (49.0 ± 2.1 h) was significantly longer than that of WT littermates (36.0 ± 2.6 h; $p = 0.0047$; Fig. 6).

Neutrophil recruitment following* P. aeruginosa *infection. Following intratracheal infection with 10^7 CFU of *Pseudomonas aeruginosa*, total cell count and neutrophil influx were assessed in the lungs of transgenic mice and WT littermate controls. Total cell counts were significantly increased in WT and transgenic mice compared with uninfected littermates at 6 and 24 h postinfection ($p < 0.0001$; Fig. 7*A*). The percentage of neutrophils in BAL fluid was significantly higher in transgenic mice than in WT littermates (61.5 ± 5.6 vs 29.9 ± 7.2; $p = 0.014$) at 6 h postinfection; however, at 24 h postinfection, the percentage of neutrophils in the BAL fluid was higher in WT mice (88.9 ± 1.9 vs 63.7 ± 8.0; $p = 0.04$; Fig. 7*B*).

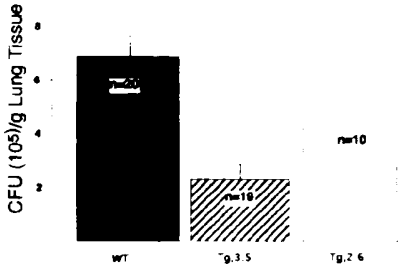

FIGURE 4. Killing of GBS at 6 h postinfection is enhanced in trans-

of animals per experiment. $p = 0.01$ for WT vs transgenic, $p = 3.5$ and 0.04 for WT vs transgenic line 2.6.

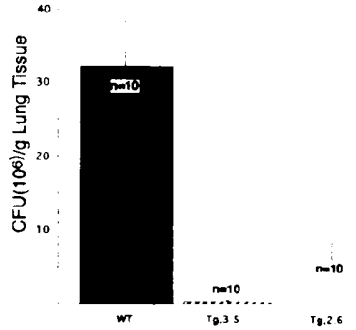

FIGURE 5. Killing of *P.* [illegible] at 24 h is [illegible]

[illegible] animals per experiment. $p = 0.03$ for WT vs transgenic line 3.5; $p = 0.05$ for WT vs transgenic line 2.6.

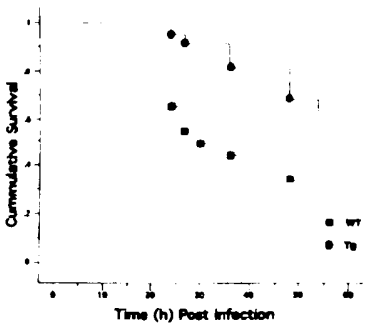

FIGURE 6. Survival following infection with *P. aeruginosa* is enhanced in transgenic (Tg) mice. *P. aeruginosa* (2×10^7 CFU) was injected into the trachea of 20 5-wk-old transgenic mice and 20 age-matched WT littermates from line 3.5 as described in *Materials and Methods*. The number of surviving mice was counted every 12 h for up to 60 h. The mean survival time for transgenic mice was significantly longer than the survival time for WT littermates (49.0 ± 2.1 vs 36.0 ± 2.6 h), $p = 0.0047$. ■, WT mice; ●, transgenic mice.

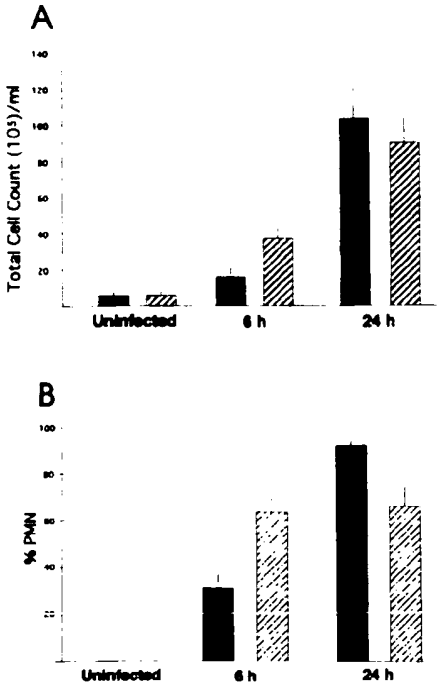

FIGURE 7. Recruitment of neutrophils into the airspace is enhanced in transgenic mice early in *P. aeruginosa* infection. *P. aeruginosa* (10^7 CFU) was injected into the trachea of five 5-wk-old transgenic mice and five age-matched WT littermates from transgenic line 3.5. Total cell counts and differential counts were assessed on the BAL fluids at 6 and 24 h as described in *Materials and Methods*. Compared with uninfected mice, total cell counts were significantly elevated in both groups at 6 and 24 h ($p < 0.0001$; *A*). The percentage of neutrophils in BAL fluid from transgenic mice was significantly higher in transgenic mice at 6 h following infection ($p = 0.014$), whereas at 24 h postinfection, the percentage of neutrophils was higher in WT mice ($p = 0.04$; *B*). ■, WT mice; ▨, transgenic mice.

Analysis of proinflammatory mediators following infection. The levels of four proinflammatory cytokines assessed following infection with *P. aeruginosa* were significantly elevated at both 6 and 24 h compared with those in uninfected littermates of either genotype ($p < 0.02$); uninfected mice had little or no detectable proinflammatory mediators in BAL fluid and lung tissue. Levels of mMIP-2 in BAL fluid and lung tissues were significantly higher in transgenic mice than in WT littermates 6 h after intratracheal infection with *P. aeruginosa* ($p = 0.03$ for lung homogenate; $p = 0.04$ for BAL fluid; Fig. 8); however, at 24 h postinfection there was no significant difference between transgenic mice and WT littermates. There were no significant differences in the levels of mouse (m) TNF-α, mIL-6, and mRANTES between transgenic mice and control littermates at 6 and 24 h postinfection (data not shown).

Discussion

Rat lysozyme was expressed in the respiratory epithelium of transgenic mice, increasing lysozyme protein levels and enzyme activity in BAL fluid without altering lung structure or function. Increased lysozyme was associated with enhanced bacterial killing and decreased mortality following infection with *P. aeruginosa*, a major human airway pathogen. Lysozyme also conferred protection against GBS, although the effect was not as pronounced as that against *Pseudomonas*. While other pathogens were not tested, the transgenic mice generated in this study should provide a useful animal model to begin to delineate the spectrum of lysozyme antimicrobial activity in the lung.

The spectrum of lysozyme antimicrobial activity in vitro appears to be relatively narrow, leading some investigators to conclude that exogenous lysozyme would be of little benefit in controlling bacterial infection (20). However, it is important to note that the results of in vitro studies are not necessarily predictive of lysozyme activity in vivo because of potential synergistic actions

[illegible] is synergistic in combination with lactoferrin is bactericidal for several Gram-negative strains (22). In the current study the dis- [illegible] proportional increase in enzyme activity relative to lysozyme transgene protein may also be the result of such synergy. These results underscore the importance of testing the antimicrobial activity of lysozyme in vivo where numerous synergistic interactions are possible.

Several mechanisms have been proposed for the microbicidal activity of lysozyme in vitro. Lysozyme is a muramidase whose enzymatic activity is typically assessed by hydrolysis of the glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine in the cell wall of *Micrococcus lysodeikticus*. Muramidase activity in BAL of transgenic mice expressing rat lysozyme was increased 17-fold relative to that in WT mice, suggesting that elevated lysozyme enzyme activity may account for much of the enhanced clearance of *P. aeruginosa*. However, there is also evidence that lysozyme possesses antibacterial activity that is independent of muramidase activity. Enzymatically inactive lysozyme was shown to retain bactericidal activity (23, 24), and During et al. (25) recently demonstrated that small amphipathic

[illegible] These results are consistent with the [illegible] bactericidal action of lysozyme against some bacteria

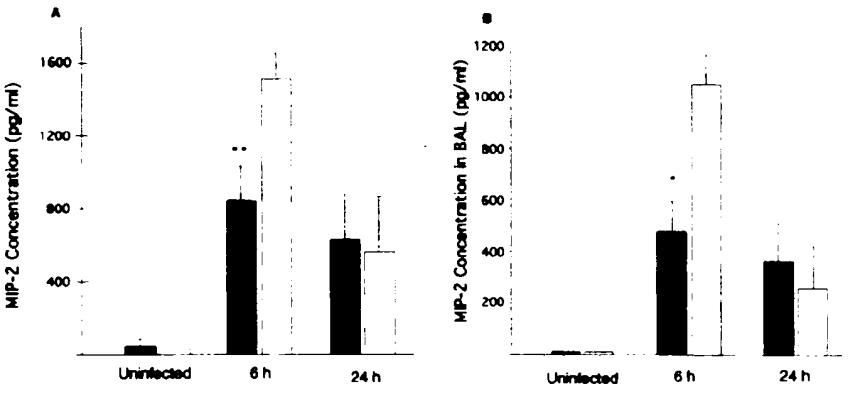

FIGURE 8. Levels of mMIP-2 are significantly increased in transgenic mice 6 h postinfection. *P. aeruginosa* (10^7 CFU) was injected into the trachea of eight 5-wk-old transgenic mice and eight age-matched control WT littermates from transgenic line 3.5. The levels of proinflammatory mediators were assessed in BAL fluid and lung tissues at 6 and 24 h postinfection and in an equal number of uninfected littermates as described in *Materials and Methods*. Levels of mTNF-α, mIL-6, and mRANTES were not significantly different between transgenic mice and control WT littermates. Levels of mMIP-2 were significantly higher in transgenic mice at 6 h in both BAL ($p = 0.04$; *B*) and lung homogenates ($p = 0.03$; *A*). ■, WT mice; □, transgenic mice.

Enhanced bacterial killing in transgenic mice may be partly due to increased recruitment of neutrophils early in the course of infection. Neutrophil influx into the airspaces was slightly, but significantly, higher in transgenic mice at 6 h postinfection, further, the level of mMIP-2, a neutrophil chemotactic factor, was significantly elevated in transgenic mice at this time point. In contrast, neutrophil numbers and mMIP-2 levels in unchallenged transgenic mice were not different from those in WT littermates. These data suggest that constitutive overexpression of lysozyme in the airways may enhance neutrophil recruitment following infection. Although it is reasonable to speculate that increased neutrophil influx may contribute to enhanced bacterial killing in these transgenic mice, this hypothesis has not been directly tested.

Lysozyme was previously identified in rat alveolar type II epithelial cells by immunohistochemistry (5, 6). In the present study lysozyme was amplified by PCR using cDNA generated from highly purified isolated rat type II epithelial cells; the deduced amino acid sequence of the amplified product was identical with the previously reported sequence for rat lysozyme (26) with the single exception of a substitution of glycine for tryptophan at position 46. Immunogold labeling and subcellular fraction of type II epithelial cells localized lysozyme to lamellar bodies, secretory granules in which pulmonary surfactant is stored (27–29). Following secretion, lysozyme was detected in association with tubular myelin, a lattice-like network of surfactant membranes (27, 28). Interestingly SP-A, another lamellar body protein involved in airway host defense (30, 31), was also associated with tubular myelin (28, 32), raising the possibility that this structure may serve as a scaffold for host defense proteins involved in alveolar surveillance.

Although the present study has not defined the precise mechanism by which lysozyme promotes bacterial killing in vivo, these results have important implications for enhancing airway host defense. Lysozyme levels in transgenic mice were constitutively elevated without affecting lung structure, suggesting that lysozyme could be safely administered for extended therapy. Most importantly, elevated levels of lysozyme significantly enhanced killing of *P. aeruginosa*, the most common pathogen associated with chronic colonization of the airway in cystic fibrosis patients. Ly-

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The role of antimicrobial peptides in animal defenses

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It is becoming clear that the cationic antimicrobial peptides are an important component of the innate defenses of all species of life. Such peptides can be constitutively expressed or induced by bacteria or their products. The best peptides have good activities vs. a broad range of bacterial strains, including antibiotic-resistant isolates. They kill very rapidly, do not easily select resistant mutants, are synergistic with conventional antibiotics, other peptides, and lysozyme, and are able to kill bacteria in animal models. It is known that bacterial infections, especially when treated with antibiotics, can lead to the release of bacterial products such as lipopolysaccharide (LPS) and lipoteichoic acid, resulting in potentially lethal sepsis. In contrast to antibiotics, the peptides actually prevent cytokine induction by bacterial products in tissue culture and human blood, and they block the onset of sepsis in mouse models of endotoxemia. Consistent with this, transcriptional gene array experiments using a macrophage cell line demonstrated that a model peptide, CEMA, blocks the expression of many genes whose transcription was induced by LPS. The peptides do this in part by blocking LPS interaction with the serum protein LBP. In addition, CEMA itself has a direct effect on macrophage gene expression. Because cationic antimicrobial peptides are induced by LPS and are able to dampen the septic response of animal cells to LPS, we propose that, in addition to their role in direct and lysozyme-assisted killing of microbes, they have a role in feedback regulation of cytokine responses. We are currently developing variant peptides as therapeutics against antibiotic-resistant infections.

Animals are exposed to millions of potential pathogens daily, through contact, ingestion, and inhalation. Their ability to avoid infection depends on their mechanisms of innate immunity. There has been a tendency to emphasize the role of the humoral and or cellular immunological system in defense against infection; however, it is equally clear that this system is not triggered rapidly enough to protect against exposure to pathogens. In the past decade, the role of cationic antimicrobial peptides has become increasingly apparent (1), and there is a growing body of evidence that their role in defense against microbes is as important to the host as antibodies, immune cells, and phagocytes. In the fruit fly *Drosophila*, for example, cationic peptides are the major form of defense against infection and are induced, in response to challenge by bacteria or lipopolysaccharide (LPS), by a regulatory pathway similar to that used by the mammalian immune system, involving Toll receptors and the transcription factor NF-κB (2).

The Nature of Cationic Antimicrobial Peptides

We use the term (cationic) antimicrobial peptides to describe gene-encoded peptides comprising between 12 and 50 amino acids, with at least two excess positive changes due to lysine and [illegible]

β-strands stabilized by disulphide bridges, amphipathic α-helices, extended structures, and loop structures (Table 1). Despite these different folding patterns, there appear to be two types of three-dimensional configurations, an amphipathic structure with opposing hydrophobic and polar cationic faces and a cationic double-wing structure with two regions of positive charge bracketing a hydrophobic core [ref. 1 and A. Rozek, C. Friedrich & R.E.W.H. (unpublished results)].

Examples of mammalian cationic antimicrobial peptides are presented in Table 1. In addition to these classes, there is a variety of animal cationic proteins including bactericidal permeability increasing protein, lactoferrin, transferrin, cathepsin G, cystatin, CAP18, pepsinogen C, ribosomal protein S30, etc., whose antibacterial activities can be traced to a cationic peptide sequence within these basic proteins.

These peptides are termed antimicrobial because they have unusually broad spectra of activity. These can include an ability to kill or neutralize Gram-negative and Gram-positive bacteria, fungi (including yeasts), parasites (including planaria and nematodes), cancer cells, and even enveloped viruses like HIV and herpes simplex virus. Nevertheless, many peptides are quite selective for microbes over eukaryotic cells. Not all peptides have all of the above activities. However, a single 13-aa peptide, indolicidin, for example, is able to kill bacteria, fungi, and HIV.

Antimicrobial peptides tend to be found in those parts of animals that are most likely to come into contact with pathogens from the environment. Thus, they are found on the skin, ear, and eye, on epithelial surfaces, including the tongue, trachea, lungs, and gut, and in the bone marrow and testes. They are also the most prevalent protein species of neutrophils, being associated with azurophilic granules and comprising a major nonoxidative killing mechanism of these dedicated antimicrobial phagocytes.

Mechanism of Production in Animals

The mucous layer of animals covers and protects all epithelial tissues against microbial, mechanical, and chemical insults and forms an initial line of defense. The main constituents are the mucins and other glycoproteins, proteinase inhibitors, and cationic peptides, like defensins. Healthy-tissue epithelial cells have been shown to express β-defensin genes at a low level. However, some defensin genes can be induced on treatment with proinflammatory cytokines, LPS, or bacteria. For example, production of human β-defensin (HBD)-2 in keratinocytes is strongly induced on contact of keratinocytes with Gram-negative bacteria or proinflammatory cytokines such as tumor necrosis factor (TNF)-α or IL-1β (4). HBD-2 mRNA expression has been observed in the skin, foreskin, lungs, and trachea, but not in the

Table 1. Some representative mammalian peptides

Peptide	Class	Amino acid sequence*†
HNP-1 (α-defensin)	β-sheet	$AC_1YC_2RIPAC_3IAGERRYGTC_3IYQGRLWAFC_2C_1$
HBD-2 (β-defensin)	β-sheet	MRVLYLLFSFLFIFLMPLPGVFGGIGDPVTC$_1$LKSGAIC$_2$HPVFC$_3$PRRYKQIGTC$_2$GLPGTKC$_1$C$_3$KKP
Protegrin	β-sheet	$RGGRLC_1YC_2RRRFC_1VC_2VGR$
Indolicidin	Extended	ILPWKWPWWPWRR-NH_2
Bac5	Extended	RFRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPPFRPPLGPFP
Bactenicin	Loop	$RLC_1RIVVIRVC_1R$
LL-37	α-helical	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES
Cecropin P1	α-helical	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR

*Single-letter amino acid code.
†Subscript numbers represent amino acids that are joined by cysteine disulfides.

kidney, salivary glands, small intestine, and liver. This is in contrast to HBD-1, which is constitutively expressed mainly in the urogenital tract and kidney. Several other inducible β-defensins are produced in the epithelia, including tracheal antimicrobial peptide (TAP), isolated from bovine respiratory mucosa, lingual antimicrobial peptide, and the enteric β-defensin (5). TAP is expressed in columnar epithelial cells of the conducting airways, and the mRNA of this β-defensin peptide is upregulated in response to LPS. The production of HBD-2 by human epithelium resembles the ancient defense mechanisms of plants and insects (2), because there is evidence in these situations for the involvement of the transcription factor NFκB and Toll receptors. Such a mechanism elicits an immediate antimicrobial response to the same microorganisms that have had contact with the epithelial cells, and these responses are related to, but completely independent of, the leukocyte-dependent immune defense mechanisms. Other obvious differences between the cationic antimicrobial peptide response and the immune response in animals are the highly specific nature of immune responses, the relative slowness of immune responses because of the requirement for clonal cell expansion, and the self vs. nonself discrimination built into the immune response. In contrast, inducible peptide responses result in an ability to act against a wide spectrum of pathogens, occur within minutes rather than days, and lack real-self vs. nonself discrimination (although the peptides appear to have relatively low activity against host cells).

Evidence for Their Role in Host Defenses

The evidence for a role of cationic antimicrobial peptides in innate host defenses has become quite convincing. It includes data demonstrating that some peptides are inducible by bacterial products and convincing animal model and transgenic animal experiments indicating that the peptides protect against infection in experimental animals. The inducibility of peptides has been summarized above. The kinetics of induction are highly suggestive of a role in early defenses against infection.

The animal model data demonstrating protection by a variety of different peptides applied both topically and systemically were summarized recently (6). Such studies have confirmed the antibacterial (vs. both Gram-negative and Gram-positive bacteria), antifungal, synergistic and antiendotoxic (see below) nature of antimicrobial peptides. These results have been confirmed by studies showing systemic protection by nisin against *Streptococcus pneumoniae* infections of mice (7), local protection by the protegrin-related peptide IB367 against polymicrobic oral mucosatis in hamsters (8), protection against lethal *Pseudomonas aeruginosa* infections of burn-wound sites in mice by peptide D4E1 continuously at low levels by using a device called an osmotic pump.

An alternative method of doing these types of studies involves a "gene-therapy" treatment of mice with an adenovirus vector containing the DNA for the human peptide LL-37 (11). Such mice showed a dramatic increase in serum and lung LL-37 and demonstrated significantly fewer bacteria and a lower inflammatory response after sublethal challenge and a dramatic increase in resistance to endotoxin and *Escherichia coli* challenges.

Although the role of such peptides in defense against infections has been emphasized, many other intriguing properties have been ascribed to selected cationic peptides, including induction of the wound-repair proteoglycans termed Syndecans (12), stimulation of nonopsonic phagocytosis (13), chemoattraction of IL-8-stimulated neutrophils (14), and penetration of the blood–brain barrier (15).

It is well known (1) that cationic antimicrobial peptides are major components of certain phagocytic cells, especially neutrophils and alveolar macrophages. They appear to be involved in nonoxidative killing by such cells (16). Although oxidative killing of bacteria by phagocytes is often emphasized, nonoxidative killing can be very effective, because neutrophils from chronic granulomatis disease patients, which lack an oxidative response, are still able to kill most bacteria (16). Indeed, such patients are only substantially more susceptible to infections by *Burkholderia cepacia*, one of the bacteria that are naturally resistant to cationic antimicrobial peptide action (17).

Mechanism of Action

Cationic antimicrobial peptides have been described as membrane-active agents (1). This is certainly true for many peptides, but we and others have recently described data that indicate that the membrane is not necessarily the target for many, or perhaps even most, cationic peptides (3, 18–20). To summarize the proposed mechanism of action for Gram-negative bacteria, the peptides interact with and cross both cell envelope membranes and then kill cells by a multihit mechanism that involves action on more than one anionic target. The initial uptake across the outer membrane is via self-promoted uptake (3, 13) proposed by us 20 years ago to explain uptake of polycationic antibiotics like aminoglycosides and polymyxins across the outer membrane. In this mechanism, the peptides initially interact with the polyanionic surface LPS and competitively displace the divalent cations that bridge and partly neutralize the LPS. This causes disruption of the outer membrane (visualized as surface blebbing), and it is through these disrupted outer membranes that peptide molecules are proposed to pass (i.e., the peptides self promote their own uptake). Next, the peptides associate with the negatively

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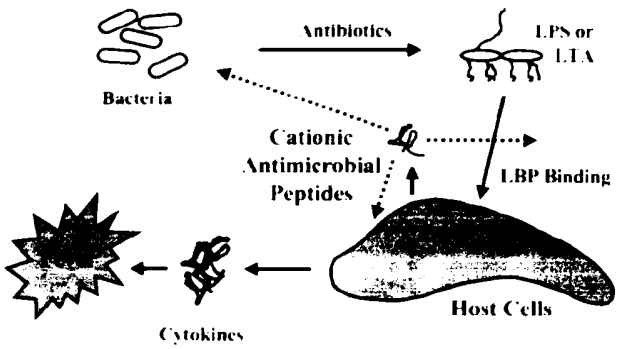

Fig. 1. Model outlining the major events in induction of sepsis by bacteria and the points at which cationic peptides are proposed to intervene.

supramolecular peptide·lipid complexes (or toroidal channels). In some cases, these channels may cause sufficiently severe permeability problems to inhibit or perhaps even kill cells. The lifetime of such channels is short (18) and, when they collapse, some of the peptide molecules are left in the inner monolayer and then are proposed to disassociate and interact with cytoplasmic polyanions such as DNA (19). Some of the potential targets in cells include DNA, membrane permeability, and autolysins. Moreover, the relative importance of each of these targets may vary from peptide to peptide.

Nevertheless, one consequence of this physical mechanism of action (based on ionic and hydrophobic interactions) is that it is rather difficult for bacteria to become resistant to such peptides, and making mutants is not at all easy (22). Mutation of the PhoP/PhoQ system is reputed to lead to resistance to cationic antimicrobial peptides but actually only increases minimum inhibitory concentrations about 2- to 4-fold. Very few naturally resistant bacteria occur; they include *B. cepacia* (which has an outer membrane composition that does not permit self-promoted uptake) and *Proteus* and *Serratia* sp., which make proteases that cleave some but not all cationic peptides.

Role in Counteracting Sepsis

More than a half million patients suffer from sepsis every year in North America. Sepsis is associated with the presence of pathogenic microorganisms or their toxins in the blood (Fig. 1). It can result from infections with either Gram-negative or Gram-positive bacteria. Gram-negative sepsis is usually caused by the release of a bacterial outer membrane component, endotoxin (LPS). The toxicity of LPS is contained within its lipid A portion. Gram-positive sepsis is also presumed to be caused by the release of bacterial cell wall components. A number of Gram-positive cell wall constituents, including lipoteichoic acid (LTA) (23), peptidoglycan (PG) (24), *Streptococcus* rhamnose-glucose polymers (25), and *Staphylococcus* capsular polysaccharide (26), have been shown to stimulate the production of inflammatory mediators *in vitro*. When injected into animals, these Gram-positive cell wall components elicit many of the characteristic features of septic shock, including cytokine production, leukocytopenia, circulatory failure, multiple organ dysfunction syndrome, and mortality (27, 28). Antibiotics used to treat the bacterial infection can actually be harmful in that they can stimulate the release of endotoxin (29), or, in the case of Gram-positive bacteria, peptidoglycan and LTA (30, 31). Both LTA and PG

dence of Gram-positive-induced septic shock (33) indicates that there is a need to develop therapeutic strategies to prevent the activation of inflammatory cells by components of Gram-positive cell walls.

The mechanism by which LPS activates macrophages is now understood in some detail (ref. 33; Fig. 1). LPS-binding protein (LBP), an acute-phase reactant that is present in the blood, binds LPS and transfers it to CD14, a protein that exists as a soluble form in blood and as a glycosyl phosphatidylinositol-linked molecule on the surface of monocytes and macrophages. LPS·CD14 complexes are thought to initiate intracellular signaling reactions by binding to Toll-like receptors (TLRs) on macrophages and other cells (33). TLR4 appears to be required for LPS to initiate signaling and to induce inflammatory responses. LPS·CD14 complexes cause activation of the NF-κB transcription factor as well as activation of the ERK, JNK, and p38 mitogen-activated protein kinases, all of which mediate the production of inflammatory cytokines (34). Despite their structural differences, both LTA and PG also activate macrophages and polymorphonuclear leukocytes in association with CD14 and TLR4/TLR2 (35–38). Thus, substances that bind to bacterial components and ablate their ability to bind surface receptors would be good candidates as antiendotoxic agents. A fragment of the cationic protein bactericidal permeability increasing protein (rBPI-21) is currently in clinical trials for this purpose (6).

As described above, cationic peptides act on Gram-negative bacteria by initially binding to their surface polyanionic LPS, followed by self-promoted uptake across the outer membrane. We have shown that some cationic peptides have a high affinity for LPS (17, 39). Coincidentally, such peptides inhibit the production of cytokines such as TNF-α and IL-6 by macrophages stimulated with LPS. We have been able to demonstrate such effects with peptides representing all structural classes, including such animal peptides as defensins, indolicidin, and protegrin (40). Furthermore, studies with the cationic proteins bactericidal permeability increasing protein, CAP37, and lactoferrin (41, 42), have indicated that these molecules have an analogous ability to antagonize the ability of LPS to stimulate cytokine production in macrophages.

We went on to study the potential *in vivo* relevance of these observations. The peptides CEMA and CEME were able to prevent lethal endotoxemia in the galactosamine-sensitized mouse model (39). With the former more active peptide, it was shown that the LPS-stimulated induction of the important sepsis-mediating cytokine, TNF-α, could also be dramatically suppressed in the blood of galactosamine-sensitized mice.

It was also possible to assess binding of cationic antimicrobial peptides to the Gram-positive surface molecule LTA by using a variation of the dansyl polymyxin displacement assay that is used to assess LPS binding (43). LTA, like LPS, has both a polyanionic and lipidic nature, and thus was able to interact with dansyl polymyxin and cationic peptides, although the kinetics of binding indicated a lower affinity for binding to LTA compared with LPS. From these studies, it appeared that the action of cationic peptides against Gram-positive bacteria did not appear to be related to their ability to bind LTA, as the relative ability of these peptides to bind LTA did not correspond to their minimum inhibitory concentration values. For example, the α-helical cecropin·melittin hybrid peptide CEME was the most effective peptide at killing *S. aureus* and other Gram-positive bacteria, even though many of the peptides studied had a higher affinity

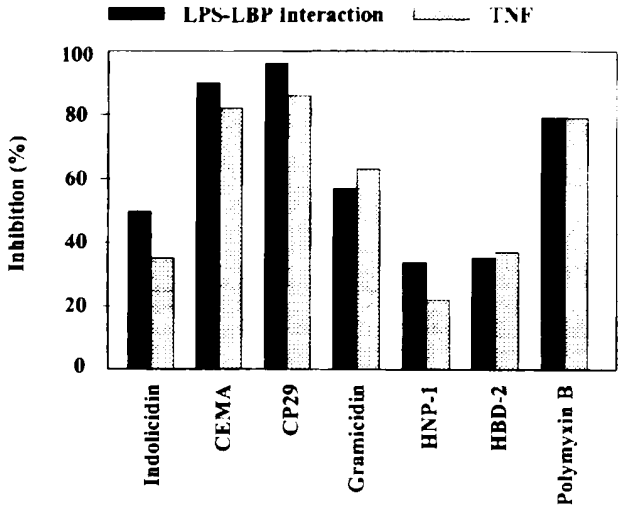

Fig. 2. Inhibition of LPS–LBP interaction and LPS-induced TNF-α production by structurally different cationic peptides. (*A*) Biotinylated LPS (45 ng/ml) was added to wells with immobilized LBP in the presence or absence of the indicated cationic peptides (10 μg ml). The peptides were added to the wells at the same time as the LPS, and residual LPS binding was assessed by ELISA. (*B*) RAW 264.7 cells were incubated with *E. coli* O55 B5 LPS (100 ng/ml) in the presence or absence of the indicated peptides (20 μg ml) for 6 h. TNF-α released into the culture supernatant was measured by ELISA. Data are from ref. 39.

were able to significantly inhibit this production of cytokines. The peptides were also effective in human blood at reducing production of TNF-α in response to LTA, although the levels of inhibition were lower than those observed in *in vitro* studies with RAW macrophage cells. The effects of the peptides on soluble LTA are significant, because bacteria release LTA during normal growth, and LTA release is enhanced by β-lactam antibiotics (31). Using a system in which macrophages are separated from bacteria by a membrane filter, we demonstrated that growing *E. coli* O111:B4, *P. aeruginosa* PAO, and *S. aureus* could, in a dose-dependent manner, stimulate the production of TNF-α, and in the case of the Gram-negative bacteria only, IL-6, because of their shedding into the medium of molecules that were able to cross the filter and interact with macrophages (39, 43). These stimulatory molecules are probably shed LPS or LTA, respectively, as indicated by the ability of cationic peptides to block cytokine production in this situation.

The mechanism of peptide blocking of the stimulation of macrophages by LPS was studied (40). Because it is known that the action of LPS is enhanced by binding to a serum protein, LBP, one possible mechanism is clearly the ability of the peptides to bind with high affinity to LPS. Indeed, in the case of LPS, the peptides can bind LPS (17, 39) and inhibit LPS-LBP interaction (Fig. 2). However, although coincubation of peptides, LPS, and LBP resulted in successful inhibition of LPS binding to LBP, the antimicrobial peptides were less successful in dissociating LPS-LBP complexes. In other experiments, it had been observed that cationic antimicrobial peptides could block the ability of LPS to stimulate TNF-α production by serum-bathed macrophages, even when added up to 1 hour after the LPS (39). This indicated that there might be another mechanism by which the peptides were acting, possibly a direct interaction with the

of such cells, by the rather low membrane potential (−15mV) across the plasma membrane of eukaryotic cells, and by the presence of cholesterol in the plasma membranes of such cells (compared to bacteria that have an abundance of anionic surface phospholipids such as phosphatidyl glycerol, a transmembrane potential of −140mV, and no cholesterol). Other studies have demonstrated directly that peptide PR-39 rapidly enters human microvascular endothelial cells (12). Moreover, it is known that some tumor cell lines (but not the macrophage cell line described here) can be killed by cationic peptides at high concentrations (44).

To investigate whether cationic antimicrobial peptides might influence sepsis through a novel mechanism involving direct interaction with macrophage cells, we chose the peptide CEMA, a peptide that becomes α-helical on contact with membranes (45). Although it is derived from insect peptides, it is a paradigm for this class of peptides and has both strong antimicrobial activity against Gram-negative bacteria and good antiendotoxic activity both with cultured macrophages and in mouse models (38). Gene array technology was used to examine the differential gene expression in RAW macrophages stimulated with LPS and the cationic peptide, CEMA [M.G.S., C. M. Rosenberger, M. R. Gold, B. B. Finlay & R.E.W.H. (unpublished results)]. A considerable number of genes were found to be up-regulated by LPS, including several already published, confirming the potential value of the gene array technology. For example, LPS was observed to affect a large number of cell-cycle mediators such as cyclins, cyclin-dependent kinases, retinoblastoma proteins, and related transcription factors, confirming the antimitogenic effects of LPS on macrophages (46). There were also several genes not found previously to be regulated by LPS, including brain factor 1 (up-regulated) DP-1, Ski, and dystroglycan (down-regulated).

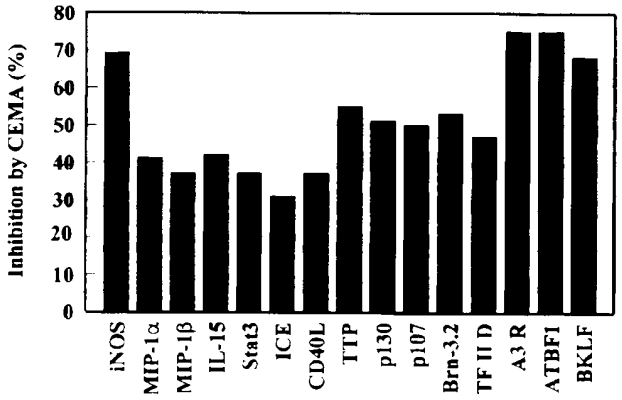

Fig. 3. Effect of CEMA on LPS induced gene expression in RAW 264.7 cells. RAW 264.7 cells were stimulated for 4 h with media alone, *S. typhimurium* LPS (100 ng ml), or *S. typhimurium* LPS (100 ng ml) and CEMA (50 μg ml). The RNA was isolated from the cells and used to make ^{32}P-labeled cDNA probes, which were hybridized to the CLONTECH Atlas arrays, and after a 3-day exposure, they were analyzed with a Phosphorimager and CLONTECH ATLAS software. The average percent inhibition of gene transcription by CEMA as measured by a change in fold intensity is shown in the graph. The following selected genes are shown: iNOS, inducible nitric oxide synthase, MIP-2 α, macrophage inflammatory protein (chemokine), MIP-1 β, macrophage inflammatory protein (chemokine), IL-15, interleukin 15; (cytokine), Stat3, acute phase response factor; ICE, interleukin

Many of the genes that showed increased expression after LPS treatment could be blocked by at least 30% by CEMA (e.g., Fig. 3). These included many known inflammatory mediators such as IL-1β, IL-15, macrophage inflammatory proteins, and inducible nitric oxide synthase. Interestingly, we saw a similar induction pattern with LTA and the bacterial DNA motif CpG, although some genes were differentially induced. The gene array data were confirmed in selective instances by assaying the stimulated macrophages for protein production in the supernatants by ELISA and by direct Northern blot analysis of transcripts. For example, the protein levels of TNF-α, IL-6, and MIP-1α were greatly increased (0.3 ng/ml to 6–12 ng/ml) by LPS stimulation, and CEMA inhibited these responses by 78, 86, and 45%, respectively (46).

In contrast, CEMA did not have a pronounced effect on LPS-induced down-regulation of gene expression. Also, CEMA did not block the ability of LPS to induce the expression of some genes, including the genes for CD14, ICAM-1, LFA-1α, HMG-box transcription factor, MAPKK1, c-*rel* proto-oncogene, and Mdm2, representing many different families of genes. This indicates that the peptides have a selective effect on gene induction by LPS. Therefore, we looked for a previously unsuspected effect of cationic peptides on macrophage gene expression. We observed that CEMA had a wide range of effects on macrophage gene expression. It up-regulated a number of genes encoding transcription factors, kinases, and cell-cycle regulators (e.g., Fig. 4), suggesting that cationic peptides not only dampen the antimitotic effect of LPS but also may directly affect cell-cycle regulation. Several cell-surface antigens and adhesion proteins were also up-regulated by CEMA. CEMA also affected several apoptosis-related genes, increasing the expression of PD-1 and decreasing the expression of BAG-1 and neuronal death protein. Thus, CEMA has pleiotropic effects on macrophages.

It appears that cationic antimicrobial peptides can suppress LPS stimulation of cytokine production in macrophages by interfering with LPS binding to serum LBP and probably other molecules receptors and possibly also by directly influencing the expression repertoire of macrophages. Because it is known that LPS may induce host-defense cationic antimicrobial peptides and do so at the same time that cationic antimicrobial peptides suppress the effects of LPS on macrophages leading to sepsis, we propose that these peptides are part of a feedback mechanism for regulating cytokine responses to bacterial products (Fig. 1).

There is substantial interest in identifying novel strategies to overcome not only sepsis but also the underlying infection. Presently, there are no effective compounds that have been proven to overcome the lethal nature of sepsis. Many new strategies, including neutralizing antibodies, soluble cytokine receptors, and various endotoxin-binding factors, have been tested with mixed results. The above-described abilities of the cationic peptides warrant further studies of their potential as part of an antisepsis treatment.

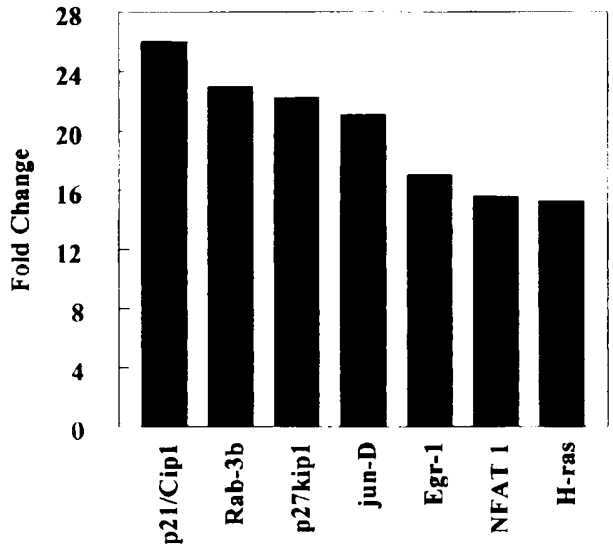

Fig. 4. Influence of CEMA on gene expression in RAW 264.7 cells. Gene arrays were used to compare transcription in unstimulated cells and cells stimulated

Peptide Lysozyme and Peptide Peptide Synergy

As discussed above, the mechanism of action of peptides leads to disruption of the outer membrane barrier and perturbation of the cytoplasmic membrane. Possibly as a consequence of these actions, antimicrobial peptides can show synergy with conventional antibiotics against both Gram-negative (17) and Gram-positive (20) bacteria. Our early studies with rabbit defensins led us to question whether peptides always acted on their own or rather in synergy with other host defense components (13). We observed that the ability of rabbit defensins to permeabilize the outer membrane of *P. aeruginosa* increased as the pH was lowered (as would occur in the phagolysosome after ingestion of *P. aeruginosa* by neutrophils), even though the antibacterial activity of defensins is antagonized at this pH. Therefore, we considered the possibility that peptides actually worked in synergy with the protein lysozyme, a slightly basic enzyme that is excluded from its target, the peptidoglycan, by the outer membrane. Indeed, we could demonstrate that peptides promoted the ability of lysozyme to lyse Gram-negative bacteria, and that there was excellent synergy between lysozyme and a range of peptides against several bacteria. Because lysozyme is present in most parts of the body, it is possible that such synergy is critical to the action of cationic antimicrobial peptides in their natural hosts. Indeed, in fish challenged with bacteria, both lysozyme and cationic peptides appear to be rapidly induced.

A second type of synergy that has been observed is between peptides. This was first demonstrated with the frog peptides magainin and PGLa (47), and we have recently confirmed this with the mammalian peptides protegrin 1 and indolicidin (unpublished results). Study of the kinetics of interaction of peptides shows that they act cooperatively. Thus, we assume that two peptides reinforce this cooperative interaction resulting in positive cooperativity.

Potential as Therapeutics

There is no question that, with the increasing antibiotic resistance problem, there is a need to develop new classes of antibiotics. Cationic antimicrobial peptides have many of the desirable features of a novel antibiotic class (6). In particular, they have a broad spectrum of activity, kill bacterial rapidly, are unaffected by classical antibiotic resistance mutations, do not easily select antibiotic resistant variants, show synergy with classical antibiotics, neutralize endotoxin, and are active in animal models. Despite this, many issues remain to be solved.

crobial peptides are very toxic for mammalian cells (e.g., bee venom melittin), whereas others show little or no acute cytotoxicity. However, more subtle toxicities have not been studied, although we assume, based on the presence of natural peptides *in vivo* at concentrations of, e.g., 44 μg ml in the saliva of an individual with peritonitis, that they can be tolerated at high levels. Another issue would be their lability to proteases in the body. In this regard, there are strategies for protecting the peptides from proteases, including liposomal incorporation or chemical modification.

With this in mind, two very promising clinical trials are underway. The protegrin derivative IB-367 (Intrabiotics, Mountain View, CA) is being examined for its potential against oral mucosaitis, a polymicrobial ulcerative disease of cancer patients. The peptide MBI-226 (Micrologix Biotech, Vancouver) is being investigated for sterilizing catheter insertion sites, thus preventing serious infections caused by colonization of such catheters by skin bacteria. Results from clinical trials to date have indicated efficacy, and MBI-226 has been given fast-track status by the Federal Drug Administration.

Thus cationic antimicrobial peptides are not only important components of the innate defenses of all animals against infections, but synthetic variants thereof hold great potential as a weapon against antibiotic-resistant bacteria. The great sequence and structural diversity offered by peptides (i.e., 20 possible amino acids in each position) will provide many possibilities for drug design.

We thank the many collaborators and laboratory personal who contributed to this work, but particularly Carrie Rosenberger, Brett Finlay, and Mike Gold, who are collaborating on transcriptional array experiments, and Hong Yan and Don Woods, who are performing the animal model infections experiments. Our peptide work is supported by the Canadian Bacterial Diseases Network and the Canadian Cystic Fibrosis Foundations SPARX program. Robert Hancock is the recipient of a Medical Research Council (MRC) of Canada Distinguished Scientist Award, and Monisha Scott has an MRC Scholarship.

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Synergistic Interactions between Mammalian Antimicrobial Defense Peptides

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A single animal can express several cationic antimicrobial peptides with different sequences and structures. We demonstrate that mammalian peptides from different structural classes frequently show synergy with each other and selectively show synergy with human lysozyme.

Cationic antimicrobial peptides are nature's antibiotics (5). They are being increasingly recognized as a component of the innate immune systems of all species of life, and more than 500 natural peptides are known. A single animal, e.g., a cow, can produce as many as three dozen antimicrobial peptides (3, http: www.univ.trieste.it ~ tossi). These can include representatives of all four known structural classes, which comprise β-sheet peptides stabilized by two to three disulfide bridges, amphipathic α-helical peptides, loop peptides, and extended peptides, in addition to peptides generated by proteolysis of larger proteins in the host.

Cationic antimicrobial peptides as a class have the ability to kill both gram-negative and gram-positive bacteria, fungi, eukaryotic parasites, and even enveloped viruses. Indeed, a single peptide can have most or all of these activities (3, 5). In addition, it has been demonstrated that such peptides can trigger the transcription of numerous genes in eukaryotic cells (3), and they appear to have multiple effects on the inflammatory response (2, 4). Given this extraordinary diversity of activities, it is worth asking why so many peptides can be observed in a single host. Possible explanations include (i) the fact that individual peptides are preferentially expressed under specific circumstances and or in specific sites in the body, (ii) the fact that different peptides cover gaps in the activity spectrum of the other peptides expressed at a given location in the body, and (iii) the fact that different peptides act in synergy with one another to reduce the concentrations required to effectively kill microorganisms. Each of these explanations is credible. Inducibility or constitutivity, as well as tissue tropism, has been demonstrated for different peptides in many host organisms (reviewed in reference 3). Similarly, it is well established that insect immunity peptides tend to be preferentially antifungal or antibacterial in nature (9). The third concept, that of synergy among individual peptides, was first observed with frog peptides, including members of the dermaseptin family (10) and the α-helical peptides magainin and PGLa (8, 13), and between β-defensins and the cationic protein BPI (7). Since then, there have been few reports demonstrating synergy between individual peptides, and generally speaking these have not addressed synergy by using the well-established methods developed by clinical microbiologists, namely, checkerboard titration.

In this paper, we examine the synergy of peptides representing each of the structural classes found in mammals against four of the more serious pathogenic bacteria in our society. In addition, we perform a limited study to examine if such synergy can also be observed between these peptides and the moderately cationic innate defense protein lysozyme.

Four of the peptides were synthesized at the University of British Columbia's Nucleic Acid and Protein Sequencing Facility using *tert*-butoxycarbonyl chemistry and were purified by reversed-phase high-pressure liquid chromatography. These were the β-hairpin pig neutrophil peptide protegrin-1, the α-helical human peptide LL-37, the loop-structured bovine neutrophil peptide bactenecin, and the extended-structure bovine neutrophil peptide indolicidin. The disulfide bonds of protegrin and bactenecin were formed by oxidation and confirmed by matrix-assisted laser desorption ionization mass spectrometry as previously described (10). The human β-defensin peptide HNP-1 was a kind gift from Bob Lehrer and Tom Ganz at the University of California at Los Angeles. Human lysozyme was purchased from Calbiochem (La Jolla, Calif.). The bacterial strains used included *Pseudomonas aeruginosa* PAO1 strain H103, *Escherichia coli* strain HB101, a methicillin-resistant *Staphylococcus aureus* (MRSA) strain, SAP0017, and *Enterococcus faecalis* ATCC 29212 (14). They were cultured on Mueller-Hinton broth, solidified when necessary with Bacto Agar (Sigma Chemical Co., St. Louis, Mo.). MIC assays and checkerboard titrations were performed using the modified microtiter dilution assay (1, 11, 14).

The peptides examined showed only modest activities (e.g., relative to the best synthetic variants [12]) against the four tested bacteria, with MICs ranging from 3.1 to 64 μg ml (Table 1). HNP-1 and human lysozyme were not active against the gram-negative bacterium *P. aeruginosa* but readily killed the gram-positive bacterium *E. faecalis*. It should be noted, however, that this represents a very stringent test of antibacterial

TABLE 1. MICs of mammalian peptides

Species	MIC (μg ml)					
	Bovine indolicidin	Pig protegrin 1	Bovine bactenecin	Human LL-37	Human HNP-1	Human lysozyme
P. aeruginosa	64	8	32	64	>50	>50
E. coli	16	4	32	4	ND[a]	ND
MRSA	8	8	16	64	ND	ND
E. faecalis	32	8	16	64	3.1	6.3

[a] ND, not done

clinically meaningful (i.e., equivalent to activity in the human host) for cationic antibiotics such as the aminoglycosides.

Synergy was measured by checkerboard titrations (1), in which one peptide is diluted along the rows of a microtiter tray and the other is diluted along the columns. In this method, one is looking for a reduction in the MIC of each compound in the presence of the other. The result is expressed as the fractional inhibitory concentration (FIC) index, which is assessed as follows: FIC = [A]/MIC_A + [B]/MIC_B, where MIC_A and MIC_B are the MICs of peptides A and B alone and [A] and [B] are the MICs of A and B when in combination. An FIC index of 0.5 is taken to indicate good synergy (representing the equivalent of a fourfold decrease in the MIC of each compound in combination). An FIC index of 1.0 represents additive activity (a twofold decrease in the MIC of each compound in combination), and an index of >4 indicates antagonism. Table 2 describes the results obtained with these peptides. In general, we observed synergy (FIC ≤ 0.5) for several combinations of peptides in *P. aeruginosa* (three of six combinations), *E. coli* (four of six combinations), and *E. faecalis* (two of six combinations) but not in any instance with MRSA. The best peptide in combination was protegrin, which showed synergy with most peptides against most bacteria. We also examined the synergies of these peptides with the human neutrophil defensin HNP-1, although the limited availability of this peptide (which has three disulfide bonds and is very difficult to synthesize) reduced the scope of these experiments to two strains of bacteria and a single concentration of HNP-1. No synergy was observed with 25 μg of HNP-1 ml and any peptide against *P. aeruginosa*. However, since we could not measure an MIC for this bacterium, we did not know what multiple of the MIC was represented by 25 μg ml and thus whether there was any possibility of seeing synergy. With *E. faecalis* we again failed to observe synergy, even though an MIC could be measured. Consistent with this, Levy et al. failed to observe synergy between defensins and a peptide, P15a (7). Nevertheless, our results overall were consistent with the conclusion that peptide-peptide synergy does occur and is peptide specific.

We also examined the synergy of peptides with human lysozyme against *P. aeruginosa* and *E. faecalis* (again, our experiments were somewhat constrained by the high cost of human lysozyme). Human lysozyme by itself is poorly antimicrobial against wild-type *P. aeruginosa* strains under the conditions tested here, causing approximately 90% killing in 60 min (15) but not demonstrating a definite MIC. A single concentration of lysozyme, 25 μg ml, consistently reduced the MICs of protegrin and bactenecin by twofold but had no effect on the MICs of indolicidin, LL-37 (Table 3), or HNP-1 (data not shown). It is possible that better synergy would have been observed if higher lysozyme concentrations were available, since we have observed synergy of many peptides with hen egg white lysozyme, which is relatively inexpensive and thus far more available (R. Hancock, unpublished data). In contrast, lysozyme showed higher natural activity and good synergy with two peptides against *E. faecalis* (Table 3), with protegrin leading the way. In checkerboard titrations, MICs of protegrin and lysozyme were lowered from 16 and 6.3 μg ml alone to 4 and 0.39 μg ml, respectively, in combination.

Of the peptides studied in detail, only indolicidin and bactenecin from cattle actually coexist in nature. A major reason that we chose the studied peptides was that information on the types of peptides in any given species remains quite fragmentary, with defensins as the major structural type identified in mammals (3, 5). Therefore, in this study we chose peptides primarily to represent the individual structural classes, which differ maximally from one another. It is thus an assumption here that peptides of given structural classes, regardless of their mammalian origin, will act in similar ways. Consistent with this, we have observed synergy similar to that observed

TABLE 2. Synergy among peptides expressed as the FIC

Species	Lowest FIC index ([A]/[B])[a]					
	Indolicidin + protegrin 1	Indolicidin + bactenecin	Indolicidin + LL-37	LL-37 + protegrin 1	LL-37 + bactenecin	Bactenecin + protegrin 1
P. aeruginosa	0.25 (8/1)	0.75 (16/16)	1.0 (32/64)	0.31 (4/1)	1.0 (32/16)	0.50 (8/2)
E. coli	0.25 (2/1)	0.5 (4/8)	0.75 (3/6)	0.31 (1.25/0.5)	0.56 (3/2)	0.50 (8/2)

TABLE 3. Synergy of peptides with human lysozyme against *E. faecalis*

Bacterium	Peptide	MIC (μg/ml)				FIC index
		Peptide alone	Lysozyme alone	Peptide in combination	Lysozyme in combination	
E. faecalis	Indolicidin	128	6.3	8	3.1	0.56
	Protegrin	16	6.3	4	0.39	0.31
	LL-37	128	6.3	32	3.1	0.75
	Bactenecin	32	6.3	8	1.6	0.5
P. aeruginosa	Indolicidin	128	>50	128	25[a]	>1.0
	Protegrin	32	>50	16	25	0.75
	LL-37	128	>50	128	25	>1.0
	Bactenecin	128	>50	64	25	0.75

[a] Due to the high cost of lysozyme, a single concentration of lysozyme (25 μg/ml) was used for these experiments.

with LL-37 with an insect-derived α-helical peptide (R. Hancock, unpublished observations).

Together, these data are consistent with the proposal that antimicrobial peptides demonstrate synergy with each other and with lysozyme in a peptide-specific manner. It is assumed that this reflects the cooperative interactions of the peptides with the outer membranes of gram-negative bacteria and/or cooperative interaction with lipid bilayers in general (8, 15). We conclude, therefore, that given the substantial diversity of peptides in any given location in the host, synergistic interactions are an important determinant of the overall effectiveness of the peptides.

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